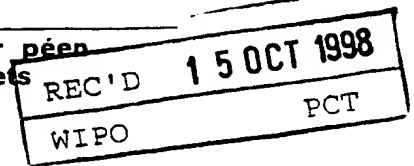**Eur päisches Patentamt**

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Patentanmeldung Nr. **Patent application No.** **Demande de brevet n°**

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Novel method of detecting amyloid-like fibrils or protein aggregates

The present invention relates to methods of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on filters. Preferably, said fibrils or aggregates are indicative of a disease, preferably of a neurodegenerative disease such as Alzheimer's disease or Huntington's disease. In addition, the present invention relates to inhibitors identified by the method of the invention, to pharmaceutical compositions comprising said inhibitors and to diagnostic compositions useful for the investigation of said amyloid-like fibrils or aggregates.

Amyloid-like fibrils and aggregates are found widespread in nature. For example, protein aggregates are found as inclusion bodies in bacteria. Such inclusion bodies may in particular arise during the recombinant expression of proteins in bacteria. In addition, a variety of diseases, both in humans and animals, is characterized by the pathogenic formation of amyloid-like fibrils or protein aggregates in neuronal tissues. A well-known and typical example of such diseases is Alzheimer's disease (AD). AD is characterized by the formation of neurofibrillar tangles and β-amyloid fibrils in the brain of AD patients. Similarly, scrapie is associated with the occurrence of scrapie-associated fibrils in brain tissue.

Another class of these diseases is characterized by an expansion of CAG repeats in certain genes. The affected proteins display a corresponding polyglutamine expansion. Said diseases are further characterized by a late onset in life and a dominant pathway of inheritance.

A typical representative of this class of diseases is Huntington's disease. Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder characterized by personality changes, motor impairment and subcortical dementia (Harper, 1991). It is associated with a selective neuronal cell death occurring primarily in the cortex and striatum (Vonsattel et al., 1985). The disorder is caused by a CAG/polyglutamine (polygln) repeat expansion in the first exon of a gene encoding a large ~350 kDa protein of unknown function, designated huntingtin (HDCRG, 1993). The CAG repeat is highly polymorphic and varies from 6-39 repeats on chromosomes of unaffected individuals and 35-180 repeats on HD chromosomes (Rubinsztein et al., 1996; Sathasivam et al., 1997). The majority of adult onset cases

have expansions ranging from 40-55 units, whereas expansions of 70 and above invariably cause the juvenile form of the disease. The normal and mutant forms of huntingtin have been shown to be expressed at similar levels in the central nervous system and in peripheral tissues (Trottier et al., 1995a). Within the brain, huntingtin was found predominantly in neurons and was present in cell bodies, dentrites and also in the nerve terminals. Immunohistochemistry, electron microscopy and subcellular fractionations have shown that huntingtin is primarily a cytosolic protein associated with vesicles and/or microtubules, suggesting that it plays a functional role in cytoskeletal anchoring or transport of vesicles (DiFiglia et al., 1995; Gutekunst et al., 1995; Sharp et al., 1995) Huntingtin has also been detected in the nucleus (de Rooij et al., 1996; Hoogeveen et al., 1993) suggesting that transcriptional regulation cannot be ruled out as a possible function of this protein.

In addition to HD, CAG/polygln expansions have been found in at least six other inherited neurodegenerative disorders which include: spinal and bulbar muscular atrophy (SBMA), dentatorubral pallidoluysian atrophy (DRPLA), and the spinocerebellar ataxias (SCA) types 1, 2, 3 and 6 (referenced in Bates et al. 1997). The normal and expanded size ranges are comparable with the exception of SCA6 in which the expanded alleles are smaller and the mutation is likely to act by a different route. However, in all cases the CAG repeat is located within the coding region and is translated into a stretch of polygln residues. Although the proteins harboring the polygln sequences are unrelated and mostly of unknown function, it is likely that the mutations act through a similar mechanism. Without exception, these proteins are widely expressed and generally localized in the cytoplasm. However, despite overlapping expression patterns in brain, the neuronal cell death is relatively specific and can differ markedly (Ross, 1995), indicating that additional factors are needed to convey the specific patterns of neurodegeneration.

In the art, there is a variety of methods to determine the presence of amyloid-like fibrils or protein aggregates. For example, inclusion bodies in bacteria can be made visible microscopically. Further, amyloid-like fibrils such as from Alzheimer's disease may be analyzed by complex methodology; see, for example, Booth et al. Nature 385 (1997), 787-793, and references cited therein.

So far, however, a simple method that may be established in any laboratory without sophisticated equipment is not available. Such a method would be especially useful in routine procedures such as the testing of patient samples for amyloid-like fibrils or protein aggregates. Accordingly, the technical problem underlying the present invention was to provide a rather simple method that may routinely be used for the

detection of such fibrils or aggregates. The solution to said problem is provided by the embodiments characterized in the claims.

Thus, the present invention relates to a method of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on a filter comprising the steps of (a) contacting said filter with material suspected to comprise said fibrils or aggregates and (b) detecting whether said fibrils or aggregates are retained on said filter.

In accordance with the present invention, it has surprisingly been found that filters of a variety of consistencies may be employed to retain detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on their surface. Essentially, only the above-recited two steps are necessary in order to investigate whether said fibrils or aggregates are present in a sample. The first step comprises contacting the filter with material suspected to comprise said fibrils or aggregates. The term "suspected to comprise" is intended to mean that the investigator may start from the assumption that the material indeed contains such fibrils or aggregates. Alternatively, said term means that it is totally unclear whether the material under investigation comprises such fibrils or aggregates.

It may be appropriate to pretreat the material prior to application to the filter. For example, for the detection of inclusion bodies, it may be necessary to first lyse the bacteria and set the cytoplasmic fraction free. Also, it may be useful to pretreat the patient samples prior to application to the filter.

The detection of fibrils or aggregates that are retained on the filter (the second step) may also be effected by a variety of steps. For example, detection may be effected by Western blot techniques, if an appropriate antibody is available.

In a preferred embodiment of the method of the invention, said amyloid-like fibrils or protein aggregates are indicative of a disease.
Of particular advantage is the method of the present invention in the detection of amyloid fibrils or protein aggregates that are indicative of human diseases, particularly in routine laboratory methods. This embodiment of the method of the invention allows the rapid determination of the disease state, if any, of the patient. For example, the concentration of amyloid-like fibrils or protein aggregates in tissue may be determined by appropriate dilution series. Using automated systems, the presence of such fibrils or aggregates per se may be determined for a large number of patients on a single filter. A further advantage of the method of the invention is

that results are available rather quickly. This has also an impact on the overall cost of the detection method, in particular in routine laboratory diagnosis. Due to the simple set-up of the method of the invention, a large number of routine laboratories, for example in hospitals, can apply the method of the invention without the need to acquire expensive equipment such as electron microscopes.

Thus, particularly preferred is the method where said disease is a human disease.

Further preferred is that said disease is associated with a polyglutamine expansion.

Most preferred is that said disease is Huntington's disease, spinal and bulbar muscular atrophy, dentarorubral pallidoluysian atropy, spinocerebellar ataxia type-1, -2, -3 or -6, Alzheimer's disease, BSE, primary systemic amyloidosis, secondary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathies: Kuru, Gerstmann-Sträussler-Scheinker syndrome (GSS), familial insomnia, scrapie, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, hereditary renal amyloidosis, or Parkinson's disease.
In particular, these diseases, for which, as a rule, no simple detection method has been developed so far, can now be assessed by simple laboratory technology.

The filter used in the method of the invention may be any filter with a sufficiently small pore size (0.2 μm or smaller). Preferred is that the filter is comprised of material with low protein absorption, and particularly preferred is that the material with low protein absorption of which said filter is comprised is cellulose acetate.

A further preferred embodiment relates to a method wherein, prior to step (b), the following step is carried out:
(b')   washing said filter so as to remove detergent- or urea-soluble material.
This embodiment is particularly preferred when enhanced sensitivity of detection is required. Such an enhanced sensitivity may be necessary, for example, when the early onset of one of the above-mentioned diseases is assessed because it is envisaged that at an early stage of any of said diseases a rather low amount of fibrils or aggregates may be found in the affected tissue or cells.
Step (b') may be repeated one or several times. The person skilled in the art is in a position to determine appropriate washing conditions without further ado. Preferably, the washing buffer comprises 0.1-2% SDS, 4-8M urea, and 0.1-2% Triton X-100.

Further preferred is in the method of the invention that the detergent- or urea-soluble material is simultaneously with or subsequent to step (a), sucked through said filter.

This embodiment is particularly useful when an apparatus for dot-blotting is available. The non-desired detergent- or urea-soluble material may be easily removed applying, for example, such a dot-blot apparatus.

In another preferred embodiment of the invention, detection in step (b) is effected by an antibody, or (poly)peptide or a fragment or derivative thereof or a chemical reagent that specifically binds to said fibrils or aggregates.

As regards the antibody, or fragment or derivative thereof, it may be employed in the Western blot type assay to determine the presence of said fibrils or aggregates. Western blot technology is well-known in the art and need not be described here in any more detail.

In a further preferred embodiment of the method of the invention, said material is derived from tissues or cells of bacteria, yeast, fungi, plants, insects, animals, preferably mammals, humans, from a transgenic animal or a transgenic plant.

In accordance with the present invention, it is additionally preferred that the method further comprises the following steps:

(a')   incubating a fusion protein comprising a (poly)peptide that enhances solubility and/or prevents aggregation of said fusion protein, an amyloidogenic (poly)peptide that has the ability to self-assemble into amyloid-like fibrils or protein aggregates when released from said fusion protein and a cleavable site that separates the above-mentioned components of the fusion protein in the presence of a suspected inhibitor of amyloid-like fibril or protein aggregate formation; and

(a")   simultaneously with or after step (a'), further incubating with a compound that induces cleavage at said cleavage site.

This embodiment is particularly advantageous for elucidating the mechanism or basis of amyloid-like fibril or protein aggregate formation. Namely, it has been surprisingly found that proteins of the above composition, after cleavage, aggregate under conditions that are, e.g., described in the appended examples. Thus, fibril or aggregate formation may be monitored under varying conditions and detected by the filter assay of the present invention.

The (poly)peptide that enhances solubility and/or prevents aggregation is preferably glutathione-S-transferase, intein, thioredoxin, dihydroflate reductase, chymotrypsin inhibitor II or a functional fragment or derivative thereof.

Preferred proteins that have the ability of self-assembling to amyloid-like fibrils or protein aggregates may be selected from the group consisting or huntingtin, androgen receptor, atropin, TATA binding protein, or ataxin-1,-2,-3 or -6 or a fragment or derivative thereof, amyloid precursor protein (APP), ß-protein, an immunoglobulin light chain, serum amyloid A, transthyretin, cystatin C, ß2-microglobulin, apolipoprotein A-1, gelsoline, islet amyloid polypeptide (IAPP), calcitonin, a prion, atrial natriuretic factor (ANF), lysozyme, insulin, fibrinogen, or α-synuclein.

Incubation conditions may be determined by the person skilled in the art according to conventional procedures. The separation of the two components of the fusion protein also includes that either or both components are degraded to a certain extent. It is, however, important that the capability of the (poly)peptide that has the ability of self-assembling is not lost due to the degradation.

Preferably, the cleavable site is an enzymatically cleavable site or a chemically cleavable site or a site cleavable by intein self-cleavage in the presence of thiols.

A number of enzymes that site-specifically or non-site-specifically digest proteinaceous material is known in the art. Examples of such enzymes are factor Xa, thrombin, trypsin, endopeptidases Arg C or Lys C, proteinase K or elastase. Depending on the goal of the experiment, any or most of the known enzymes are applicable to this test. The same holds true for most of the chemical cleavage agents.

In accordance with the present invention, it is most preferred that the method of the invention further comprises, prior to step (b) and after step (a"):

(a''')   incubation with an inhibitor of said compound that induces cleavage.

This embodiment of the method of the invention allows precise incubation periods of the cleaving agent which may be useful, for example, for studying the kinetics of aggregation.

A general overview of the above-recited specific embodiments of the invention relating to the cleaving of fusion protein are detailed in Fig. 7.

It is furthermore particularly preferred that said amyloidogenic (poly)peptide comprises a polyglutamine expansion. As has been demonstrated in the appended examples, the specific embodiments relating to the cleavage of fusion proteins may be advantageously employed for determining fibril or aggregate formation of proteins that comprise polyglutamine expansions. Accordingly, this embodiment is particularly useful for the assessment of the onset or the progress of Huntington's disease,

spinal and balba muscular atrophy, dentatorubral pallidoluysian atrophy and the spinocereberal ataxia types 1, 2, 3 and 6.

Further particularly preferred is that the polyglutamine expansion comprises at least 35, preferably at least 41, more preferably at least 48 and most preferably at least 51 glutamines.

The length of the polyglutamine expansions appears to be correlated to the susceptibility of humans or animals to the above-recited diseases. In this regard, we also refer to the European patent application entitled "Novel composition and method for the detection of diseases associated with amyloid-like fibril or protein aggregate formation" filed on the same day with the European Patent Office and assigned to the same applicant. The contents of said application are specifically incorporated herein by reference.

Further preferred is in accordance with the present invention that said contacting is effected by dotting or spotting said material onto said filter.

This embodiment is particularly useful for an automated application of the invention. Additionally, the set-up associated with dot-blotting or spot-blotting allows the investigation of a large number of sample materials as well as the cost-conscious application of the method of the invention. This is due to the fact that a large number of samples can be assessed for fibril or aggregate formation on one and the same filter.

It is furthermore preferred that the filter is a filter membrane. Additionally preferred is the use of SDS as detergent or Triton X-100 for non-β-amyloid aggregates.

The invention further relates to an inhibitor identified by the method of the invention. While practically any compound class may be tested for inhibitory effects, it is preferred that said inhibitor is an antibody or a derivative or functional fragment thereof, a peptide or a chemical reagent.

Furthermore, the present invention relates to a pharmaceutical composition comprising the inhibitor of the invention and pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways,

e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

The therapeutically useful compounds identified according to the method of the invention may be administered to a patient by any appropriate method for the particular compound, e.g., orally, intravenously, parenterally, transdermally, transmucosally, or by surgery or implantation (e.g., with the compound being in the form of a solid or semi-solid biologically compatible and resorbable matrix) at or near the site where the effect of the compound is desired.

The invention further relates to diagnostic composition comprising
(i)   a fusion protein as defined in the invention.

Preferably, the diagnostic composition further comprises
(ii)   a filter as defined in the invention; and optionally
(iii) a compound that induces cleavage as defined in any one of the preceding claims; and optionally
(iv) an inhibitor of said compound of (c); and optionally
(v)   suitable buffer solutions.

The diagnostic composition of the invention may be used for a variety of purposes. For example, it may be used for detecting the presence, etiology or status of one of the above-mentioned diseases or a corresponding disease state in a patient. In addition, it may be used for the development of suitable inhibitors of the formation of amyloid-like fibrils or protein aggregates that are preferably, but not exclusively, associated with the above-recited disease states.

The figures show:

Figure 1
SDS-PAGE Analysis of Purified GST and GST-HD Fusion Proteins.

(a) Aliquots (15 ml) of eluates from the glutathione agarose column were subjected to 12.5 % SDS-PAGE and analyzed by staining with Coomassie blue R. Lanes 1-6 contain GST, GST-HD20, -HD30, -HD83 and -HD122, respectively; lane M contains molecular mass standards. (b) Proteins were transferred to nitrocellulose and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis.

Figure 2
Structure of GST-HD fusion proteins.

The amino acid sequence corresponding to exon 1 of huntingtin is boxed. Arrows labeled Xa and T indicate cleavage sites for factor Xa and trypsin, respectively.

Figure 3
Site-Specific Proteolysis of GST-HD Fusion Proteins with Trypsin and Factor Xa.

Tryptic digestions were performed at 37°C for 3 (a) or 16 h (b). Native proteins and their cleavage products were subjected to 12.5% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis. (c) Purified fusion proteins and their factor Xa and trypsin cleavage products were analyzed using the filter retardation assay. The proteins retained by the cellulose acetate and nitrocellulose membranes were detected by incubation with the anti-HD1 antibody.

Figure 4
Electron Micrographs of Native GST-HD Fusion Proteins and their Factor Xa and Trypsin Cleavage Products.

Purified GST fusion proteins were protease treated, negatively stained with uranyl acetate and viewed by electron microscopy. The undigested GST-HD51 molecules appear as a homogeneous population of small, round particles (a). Removal of the GST-tag with factor Xa results in the formation of amyloid-like fibrils and intermediate structures (b + c). After partial digestion (3 h) of GST-HD51 with trypsin, the ribbons

are associated with terminal clots (d, arrow), whereas prolonged digestion (16 h) produces ribbons without attached clots (e). Removal of the GST-tag from GST-HD20 shows no evidence for the formation of defined structures (f).

Figure 5
Birefringence of Protein Aggregates Formed by Proteolytic Cleavage of GST-HD51.

The protein aggregates were stained with Congo red. (a) Bright field, 200x; (b) Polarized light, 200x; (c) Polarized light, 100x.

Figure 6
PolyGln-Containing Protein Aggregates are Formed *in vivo*.

(a) Western blot analysis, after separation by 10% SDS-PAGE, of the nuclear (N) and cytosolic (C) protein fractions prepared from brain and kidney of an R6/2 hemizygous transgenic mouse and a littermate control. Blots were probed with anti-HD1, anti-GAPDH and anti-Fos B antibodies as indicated. (b) Detection of HD exon 1 protein aggregates formed *in vivo* using the cellulose acetate filter assay. The membrane was immunostained using the anti-HD1 antibody. (c) Ultrastructure of a neuronal intranuclear inclusion (NII). The presence of a NII in a striatal neuron of a 17 month old R6/5 homozygous mouse is shown. The NII is indicated by the large arrow and the fibrillar amyloid-like structures within the NII are indicated by two small arrows. The scale bar is 250 nm.

Figure 7

A: Purification of GST-HD fusion proteins containing polyglutamine expansions by affinity chromatography

B: Transfer of soluble GST-HD fusion protein into a microtiter plate using a pipetting robot

C: Transfer of various inhibitors into the microtiter plate using a pipetting robot

D: Transfer of a protease to the microtiter plate using a pipetting robot to start the formation of insoluble protein fibrils. Incubation of the microtiter plate at 25°C-37°C to allow fibril formation

E: Addition of a protease inhibitor to stop the cleavage reaction using a pipetting robot

F: Transfer of the reaction mixtures onto a cellulose acetate (CA) and a nitrocellulose membrane (NC) using a spotting robot or a pipetting robot

G: Washing of the CA membrane with SDS-buffer to remove soluble proteins and the NC membrane with blocking buffer

H: Detection of the proteins bound to the CA and NC membranes by Western blot analysis using a specific antibody

I: Comparison of the membranes and identification of compounds that block fibril formation using specific computer programs

The examples illustrate the invention:

Example 1:
Purification of GST-HD fusion proteins containing expanded polyglns

Exon 1 of the HD gene was isolated from genomic phage clones, derived from the normal and expanded alleles of an HD patient (Sathasivam et al., 1997), and used for the expression of GST-HD fusion proteins in *E. coli*. DNA fragments containing CAG repeats in the normal $(CAG)_{20-33}$ and expanded $(CAG)_{37-130}$ range were cloned into pGEX-5X-1 (Pharmacia), and the resulting plasmids expressing fusion proteins with 20 (GST-HD20), 30 (-HD30), 51 (-HD51), 83 (-HD83) and 122 (-HD122) glutamines, respectively, were used for protein purification. For plasmid construction lambda phage from stock 91974 (Sathasivam et al., 1997) were plated to give single plaques which were innoculated into 400 ml cultures of *E. coli* XL1-Blue MRF' (Stratagene) for DNA preparation. The DNA sequence encoding the N-terminal portion of huntingtin (exon 1), including the CAG repeats, was amplified by PCR using the following pair of primers: ES 25 (TGGGATCCGCATGGCGACCCTGGAAAAGCTGATGAAGG) corresponding to nt315-343 of the HD gene (HDCRG, 1993) and containing a BamHI site (underlined) and ES 26 (GGAGTCGACTCACGGTCGGTGCAGCGGCTCCTCAGC) corresponding to nt516-588 and containing a Sall site (underlined). Conditions for PCR were as described (Mangiarini et al. 1996). Due to instabiltity of the CAG repeat during propagation in *E. coli* , DNA preparations from individual plaques yielded different sized PCR products. Fragments of ~ 320, 360, 480, and 590 bp were gel-purified, digested with BamHI and Sall and inserted into the BamHI-Sall site of the expression vector pGEX-5X-1 (Pharmacia), yielding pCAG30, pCAG51, pCAG83 and pCAG122, respectively. pCAG20, containing 20 repeats of CAG within the cloned HD exon 1 sequence, was similarly constructed from a phage genomic clone derived from a normal allele. All constructs were verified by sequencing. After induction with IPTG, the resulting proteins were purified under native conditions by affinity chromatography on glutathione agarose. Thus, *E. coli* SCS1 (Stratagene) carrying the pGEX expression plasmid of interest was grown to an OD_{600nm} of 0.6

and induced with IPTG (1 mM) for 3.5 h as described in the manufacturer's protocol (Pharmacia). Cultures (200 ml) of induced bacteria were centrifuged at 4000 g for 20 min, and the resulting pellets were stored at -80°C. Cells were thawed on ice and resuspended in 5 ml of lysis buffer (50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.5 mg/ml lysozyme. After 45 min at 0°C, cells were sonicated with two 30 sec-bursts. Octyl-β-D-glucopyranoside was then added to a final concentration of 0.1% and the resulting lysate was clarified by centrifugation at 30,000 g for 30 min at 4°C. Cleared lysates were incubated for 1 h at 4°C with 500 μl of a 1:1 slurry of glutathione-agarose beads (Sigma) that had been washed times and resuspended in lysis buffer. The beads were poured into a small column and washed extensively with lysis buffer containing 0.1% octyl-β-D-glucopyranoside. The bound fusion protein was eluted with 2 ml of 15 mM glutathione (reduced) in lysis buffer. Typical yields were 0.5-1 mg of purified GST-HD20, -HD30 and -HD51 proteins per 200 ml of bacterial culture; yields of GST-HD83 and -HD122 were much lower, less than 10% of that obtained with the shorter fusion proteins. Protein was determined by the Bio-Rad dye binding assay using bovine serum albumin as standard. SDS-PAGE of the purified GST-HD20, -HD30, -HD51, -HD83 and -HD122 proteins revealed major bands of 42, 45, 50, 65 and 75 kDa, respectively (Fig. 1a). These bands were also detected when the various protein fractions were subjected to immunoblot analysis using the affinity purified anti-huntingtin antibody HD1 (Fig. 1b, lanes 2-6). HD1 specifically detects the GST-HD fusion proteins on immunoblots, whereas the GST-tag alone is not recognized (Fig. 1b, lane 1). For immunoblotting a bacterial plasmid encoding HD1-His, a His_6-tagged fusion protein containing residues 1-222 of huntingtin, was generated by inserting a PCR-amplified *IT-15* cDNA fragment into the pQE-32 vector (Qiagen). The fusion protein was expressed in *E. coli*, affinity-purified under denaturing conditions on Ni-NTA agarose, and injected into rabbits. The resulting immune serum was then affinity-purified against the antigen that had been immobilized on Ni-NTA agarose. The GAPDH- and Fos B-specific antisera have been described (Wanker et al., 1997; Davies et al., 1997).

Western blotting was performed as detailed (Towbin et al., 1979). The blots were incubated with 1:1000 dilutions of the indicated primary antibody, followed by an alkaline-phosphatase-conjugated secondary antibody. Color development was carried out with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates (Promega).

All recombinant proteins migrated at a size corresponding nearly to that predicted from their amino acid sequence. Interestingly, an additional high molecular weight band which remains at the top of the gel, was consistently detected in the protein

fractions with the longest polyglns (83 and 122 residues; Fig. 1a and b, lane 5 and 6). This band was most prominent on the immunoblots but was also clearly detectable in the Commassie stained gel. This immunoreactive material was often still present at the bottom of the loading slots, even after the samples had been boiled for 5 min in the presence of 2% SDS and 6 M urea prior to loading.

Example 2:
Proteolytic cleavage of GST-HD fusion proteins containing expanded polyglns

It has been shown previously that the solubility of certain proteins can be enhanced by the addition of the GST-tag (Smith and Johnson, 1988) and it was therefore of interest to determine whether the removal of the GST-tag by proteolytic cleavage would have an effect on the solubility of the polygln-containing fusion proteins. Potential factor Xa and trypsin cleavage sites within the GST-HD fusion proteins are shown in Fig. 2. Factor Xa cleaves between the GST-tag and the HD exon 1 protein whereas trypsin removes an additional 15 amino acids from the N-terminus and a single proline from the C-terminus, both proteases leaving the polygln repeat intact. The GST-HD20, -HD30 and -HD51 proteins were digested with trypsin under conditions designed to remove the GST-tag from the fusion protein without it being totally degraded. After cleavage, proteins were denatured by boiling in the presence of 2% SDS and analyzed by SDS-PAGE and immunoblotting using the anti-HD1 antibody. GST-HD20 and -HD30 cleavage yielded products migrating in a 12.5 % gel at approximately 30 and 33 kDa, respectively. In contrast, cleavage of GST-HD51 resulted in the formation of two protein products migrating at approximately 37 and 60 kDa, and an additional weak immunoreactive band on the bottom of the loading slots was also detected (Fig. 3a). This high molecular weight band was more pronounced when GST-HD51 was digested with trypsin under conditions in which the GST-tag was totally degraded (Fig. 3b). However, with proteins GST-HD20 and -HD30 this longer exposure to trypsin produced the same cleavage products as the ones seen in Fig. 3a and the high molecular weight products were not observed. Similar results were obtained with factor Xa protease and endoproteinases Arg-C and Lys-C. As regards the proteolytic cleavages, the following protocols were carried out: The GST-HD fusion proteins purified as described above were dialysed against 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and 5% (v/v) glycerol to raise the pH prior to proteolytic cleavage. The proteins were then combined with bovine factor Xa (New England Biolabs) or modified trypsin (Boehringer Mannheim, sequencing grade) in dialysis buffer containing 2 mM $CaCl_2$ at an enzyme:substrate ratio of 1:10 (w/w) or 1:40 (w/w), respectively. Incubations with factor Xa were at 25°C for 16 h. Tryptic digestions were performed at 37°C for 3 or 16 h as indicated.

Digestions were terminated by the addition of PMSF to 1 mM . The degree of proteolysis was determined by SDS-PAGE followed by staining with Coomassie blue or immunoblottting using anti-HD1 antibody.

We have developed a simple and sensitive filter assay to detect the formation of high molecular weight insoluble protein aggregates. This assay is based on the finding that the SDS-insoluble protein aggregates obtained by proteolytic cleavage of GST-HD51 are retained on a cellulose acetate filter, whereas the soluble cleavage products of GST-HD20 and GST-HD30 are not. Factor Xa or trypsin digestions of purified GST-HD fusion proteins (10 µg) were performed in a 20 µl reaction mixture as described above. Reactions were terminated by adjusting the mixture to 2% SDS and 50 mM DTT. After heating at 100°C for 5 min, aliqouts (0.5 µl) were diluted into 200 µl of 0.1% SDS and filtered through a cellulose acetate membrane (Schleicher & Schuell, 0.2 µm pore size) using a BRL dot blot filtration unit. Filters were washed with water, and the SDS-insoluble aggregates retained on the filter detected by incubation with the anti-HD1 antibody, followed by an anti-rabbit secondary antibody conjugated to alkaline phosphatase (Boehringer Mannheim). Fig. 3c shows immunoblots of cellulose acetate and nitrocellulose membranes to which the native GST-HD20, -HD30 and -HD51 proteins and their factor Xa and trypsin cleavage products have been applied. On the cellulose acetate filter, only the cleavage products of GST-HD51 were detected by the anti-HD1 antibody, indicating the formation of insoluble high molecular weight protein aggregates. In contrast, all the uncleaved GST-HD fusion proteins and their digestion products were detected on the nitrocellulose control filter. This assay was also used to detect huntingtin aggregates present in a nuclear fraction from the brain of an R6/2 hemizygous mouse and littermate control (see preparation of nuclei below).

Example 3:
Huntingtin proteins containing expanded polyglns in the pathological range aggregate to amyloid-like birefringent fibrils

Electron microscopy of negatively stained GST-HD51 fractions showed oligomeric particles with diameters of 6 to 7 nm (Fig. 4a); no higher ordered aggregates were observed. For electron microscopic observation, the native or protease-digested GST-HD fusion proteins were adjusted to a final concentration of 50 µg/ml in 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and 5% glycerol. Samples were negatively stained with 1% uranyl acetate and viewed in a Philips CM100 EM. In contrast, protein fractions obtained by proteolytic cleavage of GST-HD51 showed numerous clusters of high molecular weight fibrils and ribbon-like structures (Fig. 4b,

c, d and e), reminiscent of purified amyloids (Prusiner et al., 1983). The fibrils obtained after digestion with factor Xa showed a diameter of 10-12 nm and their length varied from 100 nm up to several micrometers (Fig. 4b and c). In the trypsin-treated samples ribbon-like structures formed by lateral aggregation of fibrils with a diameter of 7.7 nm were observed (Fig. 4d and e). After treatment with factor Xa or limited digestion with trypsin, clots of small particles were frequently detected on one or both ends of the fibrils (Fig. 4b, c and d). These clots of varying sizes and shapes were not seen when GST-HD51 was digested with trypsin under conditions in which the GST-tag is totally degraded (Fig. 4e), indicating that they contain GST. In strong contrast to GST-HD51, the GST-HD20 and -HD30 proteins did not show any tendency to form ordered high molecular weight structures, either with or without protease treatment (Fig. 4f).

The insoluble protein aggregates formed by proteolytic cleavage of GST-HD51 were isolated by centrifugation and stained with Congo red (Caputo et al., 1992) and examined under a light microscope. For light microscopy, peptide aggregates formed by trypsin digestion of purified GST-HD fusion proteins (50 µg in 100 µl of digestion buffer) were collected by centrifugation at 30,000 g for 1 h and resuspended in 10 µl of water. Samples were mixed with 0.1 volume of a 2% (w/v) aqueous Congo Red (Sigma) solution, placed on aminoalkylsilane-coated glass slides, and allowed to dry overnight under a coverslip. After removing the coverslip, excess Congo Red was removed by washing with 90% ethanol. Evaluation of the Congo Red staining by polarization microscopy was performed using a Zeiss Axiolab Pol microscope equipped with strain-free lenses and optimally aligned cross-polarizers. After staining, the protein aggregates on the glass slides were red, indicating that they had bound the dye (Fig. 5a), and when examined under polarized light a green color and birefringence were detected (Fig. 5b and c). These staining characteristics were similar to those observed for prions (Prusiner et al., 1983) and amyloids (Caputo et al., 1992).

Example 4:

Huntingtin proteins containing expanded polyglns form amyloid-like protein aggregates *in vivo*

To determine whether the amyloid-like protein aggregates formed by proteolytic cleavage of GST-HD51 *in vitro* are also present *in vivo*, nuclear protein fractions of brain and kidney were prepared from mice transgenic for the HD mutation (line R6/2) and littermate controls (Davies et al., 1997; Mangiarini et al., 1996). Nuclei from the brain or kidney of an R6/2 hemizygous mouse with a repeat expansion of

$(CAG)_{143}$ (Mangiarini et al., 1996) at ten weeks of age and littermate control were prepared as follows. Whole brain samples (80 mg) in 400 ml of 0.25 M sucrose in buffer A (50 mM triethanolamine [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) were homogenized using 15 strokes of a tight-fitting glass homogenizer. The homogenate was adjusted to a final concentration of 5 mM DTT, and centrifuged at 800 g for 15 min. The supernatant was recentrifuged at 100,000 g for 1 h, and the supernatant from this centrifugation was taken as the cytosolic fraction (fraction C). The loose pellet from the first centrifugation was homogenized, diluted to 1.2 ml with 0.25 M sucrose/buffer A, and mixed with two volumes of 2.3 M sucrose/buffer A. The mixture was then layered on top of 0.6 ml 2.3 M sucrose/bufferA in a SW60 tube and centrifuged at 124,000 g for 1 h. The pellet was harvested with a spatula, resuspended in 200 µl of 0.25 M sucrose/buffer A and again centrifuged at 800 g for 15 min. The entire procedure was carried out at 4 °C. The pelleted nuclei were resuspended to a density of $\sim 1 \times 10^7$ nuclei/ml in 0.25 sucrose/buffer A (fraction N) and stored at -80 °C. Nuclei from mouse kidney were prepared in the same way. The protein extracts were analyzed by SDS-PAGE and Western blotting using the anti-HD1 antibody (Fig. 6a). Strikingly, this antibody detected a prominent high molecular weight band in the nuclear fraction (N) prepared from R6/2 transgenic brain, very similar to the high molecular weight band obtained by proteolytic cleavage of GST-HD51 (Fig. 3b). No such immunoreactive band was detected in the nuclear fraction of brain from the littermate control and it was also absent from the corresponding cytoplasmic fractions (C). A small amount of high molecular weight material was also detected in the nuclear fraction prepared from R6/2 transgenic kidney, but was again absent from the cytoplasmic fraction. The purity of the nuclear and cytoplasmic fractions was confirmed by Western blot analysis using the anti-Fos B and anti-GAPDH antibodies. Anti-Fos B detected the transcription factor mainly in the nuclear fraction, and the enzyme GAPDH was only seen in the cytoplasmic fraction, as expected. The Western blot results were reproduced using the cellulose acetate filter assay (Fig. 6b). Using this assay, a 10-20 fold higher amount of transgene protein was detected in the nuclear fraction isolated from brain material, compared to that prepared from kidney.

The formation of NIIs has been shown to preceed the neuronal dysfunction that forms the basis of the progressive neurological phenotype observed in the R6 transgenic lines (Davies et al., 1997). These NIIs are immunoreactive for both huntingtin and ubiquitin antibodies and contain the transgene but not the endogenous huntingtin protein. Therefore, Western blot analysis using an anti-ubiquitin antibody was also performed showing the same pattern of immunoreactivity as had been observed with the anti-HD1 antibody (Fig. 6a), and indicating that the

high molecular weight transgene protein present in the nuclear fraction is ubiquitinated (data not shown).

To examine whether the NIIs containing the proteins huntingtin and ubiquitin (Davies et al., 1997) have a fibrous composition, an ultrastructural analysis was performed. Experimentally, a 17 month old R6/5 homozygous mouse ((CAG)$_{128-155}$) (Mangiarini et al., 1996) was deeply anaesthetised with sodium pentobarbitone and then perfused through the left cardiac ventricle with 35-50 ml of 4 % paraformaldehyde and either 0.5 % glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.4). The brain was removed from the skull and placed in fresh fixative overnight at 4 °C. Coronal sections (50 - 200 µm) were cut on an Oxford Vibratome (Lancer) and collected in serial order in 0.1 M phosphate buffer. After being osmicated (30 min in 1% OsO_4 in 0.1 M phosphate buffer) the sections were stained for 15 min in 0.1 % uranyl acetate in sodium acetate buffer at 4 °C, dehydrated in ethanols, cleared in propylene oxide and embedded in Araldite between two sheets of Melanex (ICI). Semi thin (1 µm) sections were cut with glass knives and stained with toluidine blue adjacent to thin sections cut with a diamond knife on a Reichert Ultracut ultramicrotome. The sections were collected on mesh grids coated with a thin formvar film, counterstained with lead citrate and viewed in a Jeol 1010 electron microscope. An electron micrograph of a NII from a 17 month old R6/5 homozygous mouse is shown in Fig. 6c. This NII (large arrow) contains high molecular weight fibrous structures which were clearly differentiated from the surrounding chromatin. The filaments were randomly oriented, 5-10 nm in diameter and often measured up to 250 nm in length (small arrows). These structures differ from those previously reported in the NIIs seen in hemizygous R6/2 mice which were far more granular in composition, with individual filamentous structures being more difficult to distinguish (Davies et al., 1997). R6/2 mice exhibit an earlier age of onset with a more rapid progression of the phenotype and do not survive beyond 13 weeks (Mangiarini et al., 1996). It is possible that the filamentous structures do not have time to form in the R6/2 mice.

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CLAIMS

1. A method of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on a filter comprising the following steps:
    (a) contacting said filter with material suspected to comprise said fibrils or aggregates; and
    (b) detecting whether said fibrils or aggregates are retained on said filter.

2. The method of claim 1 wherein said amyloid-like fibrils or protein aggregates are indicative of a disease.

3. The method of claim 2 wherein said disease is a human disease.

4. The method of claim 2 or 3 wherein said disease is associated with a polyglutamine expansion.

5. The method of any one of claims 2 to 4 wherein said disease is Huntington's disease, spinal and bulbar muscular atrophy, dentarorubral pallidoluysian atrophy, spinocerebellar ataxia type-1, -2, -3 or -6, Alzheimer disease, BSE, primary systemic amyloidosis, secondary systemic amyloidosis, senile systemic amyloidosis, familial amyloid polyneuropathy I, hereditary cerebral amyloid angiopathy, hemodialysis-related amyloidosis, familial amyloid polyneuropathy III, Finnish hereditary systemic amyloidosis, type II diabetes, medullary carcinoma of the thyroid, spongiform encephalopathies: Kuru, Gerstmann- Sträussler-Scheinker syndrome (GSS), familial insomnia, scrapie, atrial amyloidosis, hereditary non-neuropathic systemic amyloidosis, injection-localized amyloidosis, hereditary renal amyloidosis, or Parkinson's disease.

6. The method of any one of claims 1 to 5 wherein said filter is comprised of material with low protein adsorption.

7. The method of claim 6 wherein said material with low protein adsorption is cellulose acetate.

8. The method of any one of claims 1 to 7 wherein, prior to step (b), the following step is carried out:

(b')   washing said filter so as to remove detergent- or urea-soluble material.

9.   The method of any one of claims 1 to 8 wherein detergent- or urea-soluble material is simultaneously with or subsequent to step (a), sucked through said filter.

10.   The method of any one of claims 1 to 9 wherein detection in step (b) is effected by an antibody, or (poly)peptide or a fragment or derivative thereof or a chemical reagent that specifically binds to said fibrils or aggregates.

11.   The method of any one of claims 1 to 10 wherein said material is derived from tissues or cells of bacteria, yeast, fungi, plants, insects, animals, preferably mammals, humans, from a transgenic animal or a transgenic plant.

12.   The method of any one of claims 1 to 10 further comprising the following steps prior to step (a):
(a')   incubating a fusion protein comprising a (poly)peptide that enhances solubility and/or prevents aggregation of said fusion protein, an amyloidogenic (poly)peptide that has the ability to self-assemble into amyloid-like fibrils or protein aggregates when released from said fusion protein and a cleavable site that separates the above-mentioned components of the fusion protein in the presence of a suspected inhibitor of amyloid-like fibril or protein aggregate formation; and
(a")   simultaneously with or after step (a'), further incubating with a compound that induces cleavage at said cleavage site.

13.   The method of claim 12 wherein said cleavable site is an enzymatically cleavable site or a chemically cleavable site or a site cleavable by intein self-cleavage in the presence of thiols.

14.   The method of claim 12 or 13 further comprising, prior to step (b) and after step (a"):
(a"')   incubation with an inhibitor of said compound that induces cleavage.

15.   The method of any one of claims 12 to 14 wherein said amyloidogenic (poly)peptide comprises a polyglutamine expansion.

16. The method of any one of claims 4 to 15 wherein said polyglutamine expansion comprises at least 35, preferably at least 41, more preferably at least 48 and most preferably at least 51 glutamines.

17. The method of any one of claims 1 to 16 wherein said contacting is effected by dotting or spotting said material onto said filter.

18. The method of any one of claims 1 to 17 wherein said filter is a filter membrane.

19. The method of any one of claims 1 to 18 wherein said detergent is SDS or Triton X-100.

20. An inhibitor identified by the method of any one of claims 12 to 18.

21. The inhibitor of claim 20 which is an antibody or a derivative or functional fragment thereof, a peptide or a chemical reagent.

22. A pharmaceutical composition comprising the inhibitor of claim 20 to 21 and a pharmaceutically acceptable carrier and/or diluent.

23. A diagnostic composition comprising
    (i)   a fusion protein as defined in any one of the preceding claims.

24. The diagnostic composition of claim 23 further comprising
    (ii)   a filter as defined in any one of the preceding claims; and optionally
    (iii) a compound that induces cleavage as defined in any one of the preceding claims; and optionally
    (iv)   an inhibitor of said compound of (c); and optionally
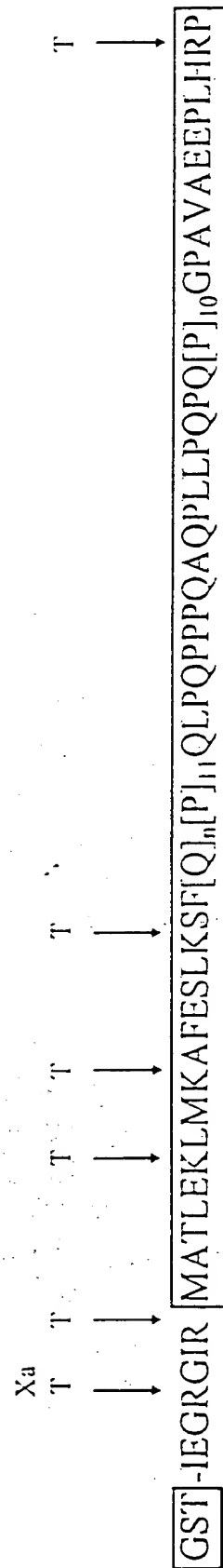   (v)   suitable buffer solutions.

41.

Fig. 1

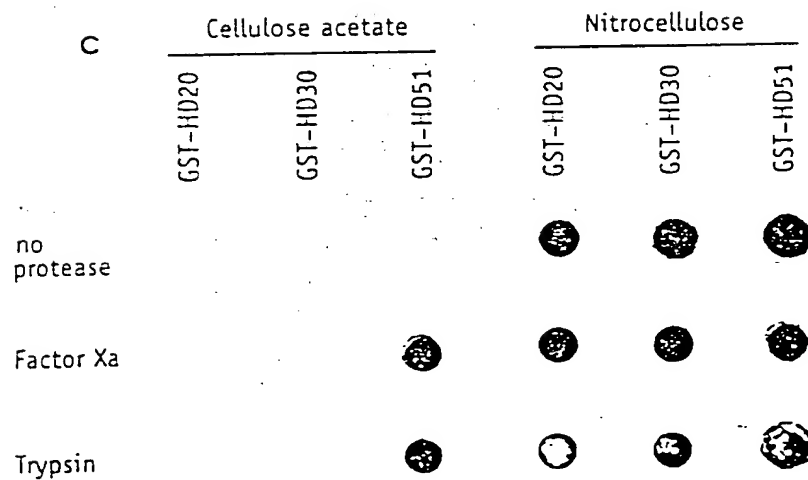GST -IEGRGIR MATLEKLMKAFESLKSF[Q]$_n$[P]$_{11}$QLPQPPPQAQPLLPQPQP[P]$_{10}$GPAVAEEPLHRP
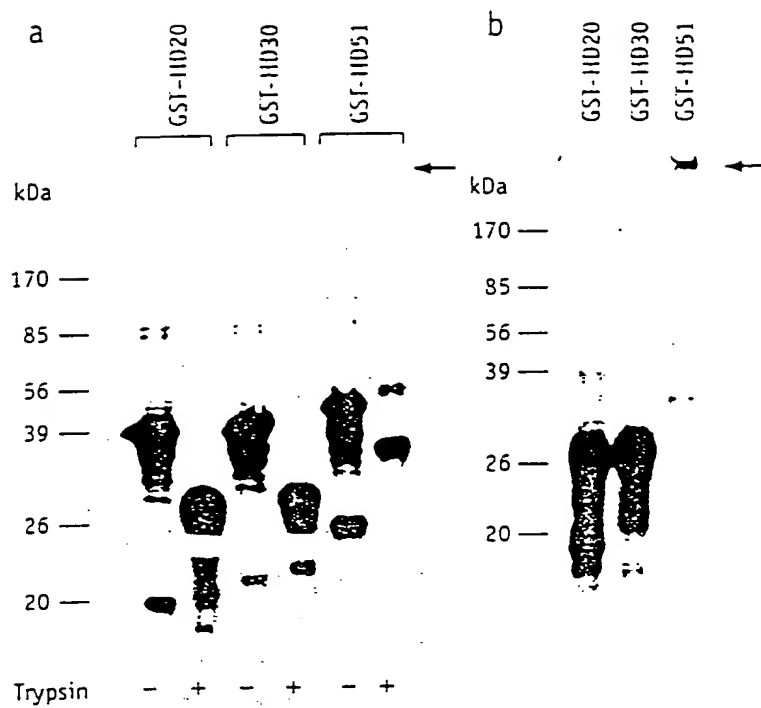
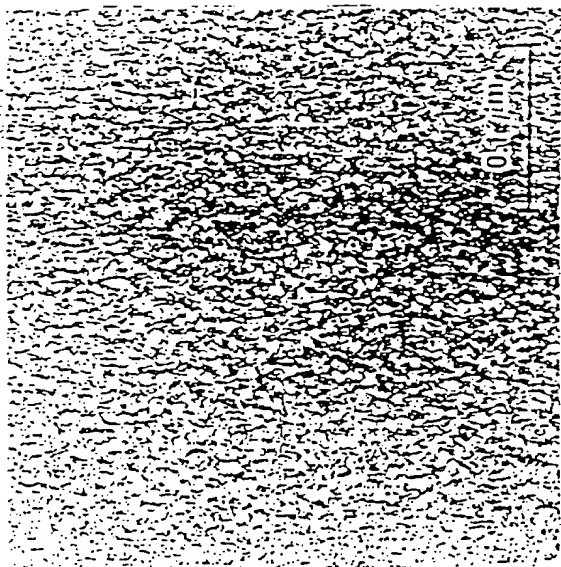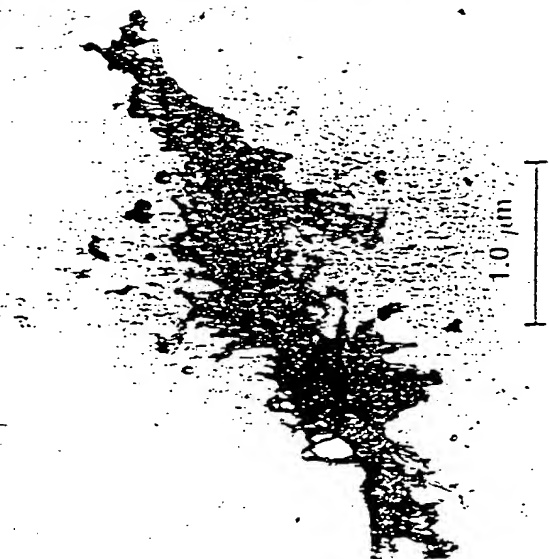Xa
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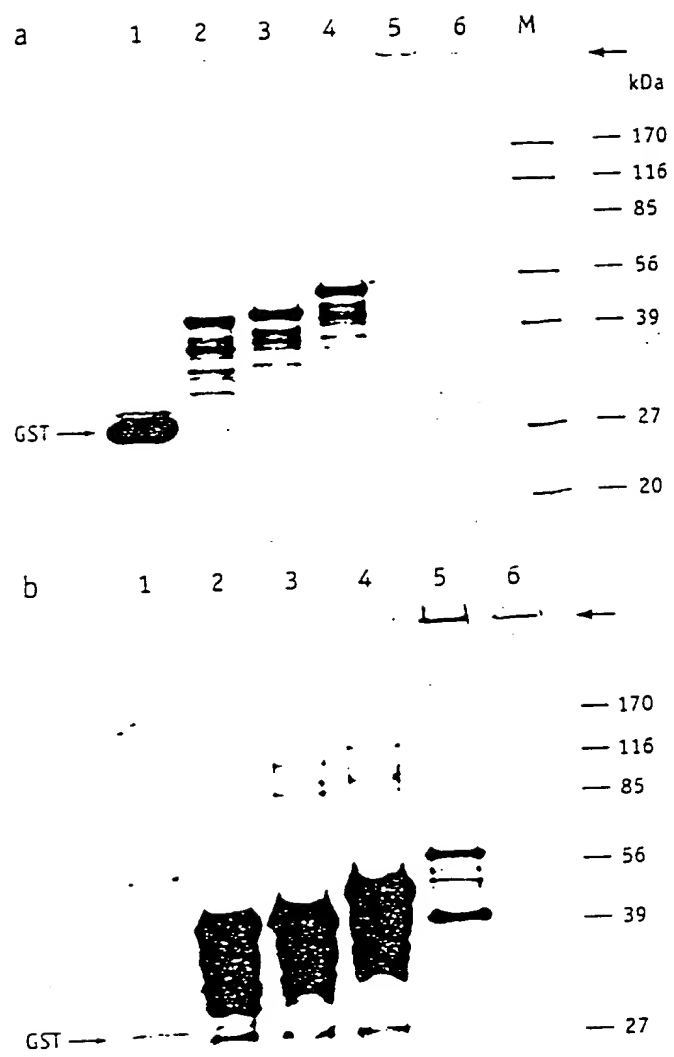
Fig. 2

Fig. 3
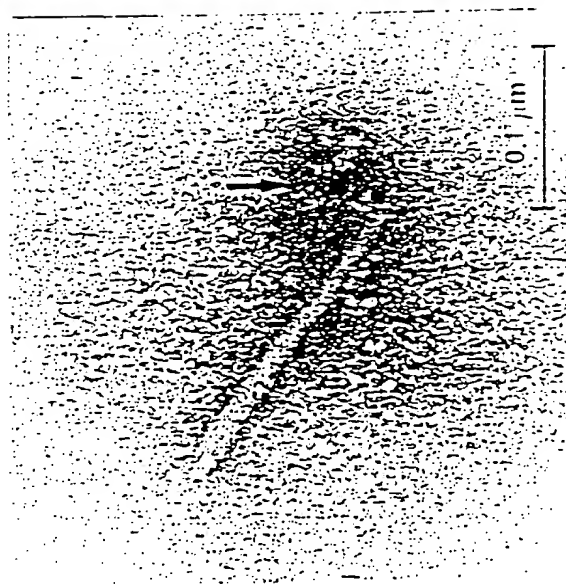
a   GST-HD51 undigested

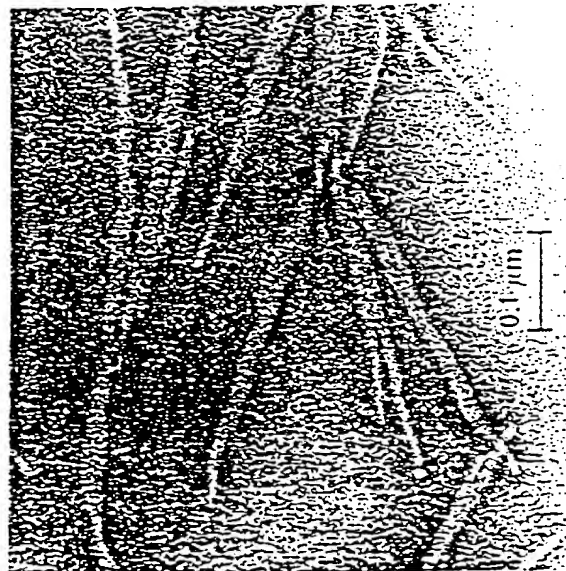b   GST-HD51 + Factor Xa

c   GST-HD51 + Factor Xa

d   GST-HD51 + Trypsin

e   GST-HD51 + Trypsin

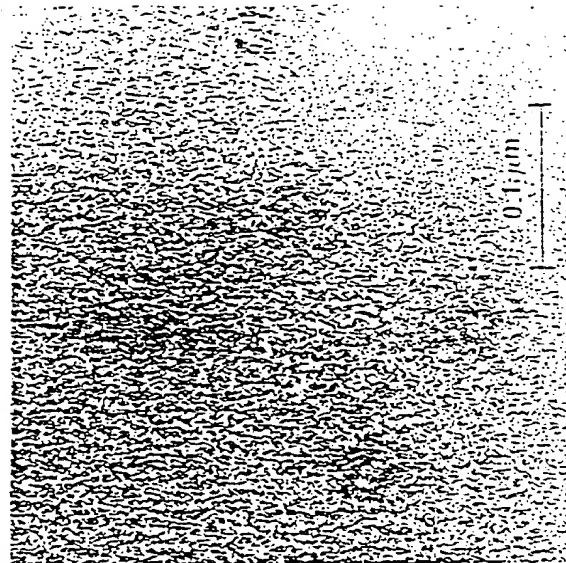f   GST-HD20 + Factor Xa

Fig. 4

Fig. 5

Fig. 6a

Fig. 6b

Fig. 6c

Fig. 7

purification of
GST-HD
protein

A

soluble
GST-HD
protein

B

inhibitors

C

D

E

site-specific
protease

protease
inhibitor

F

NC

CA

G'

G

NC

CA

H

H

NC

CA
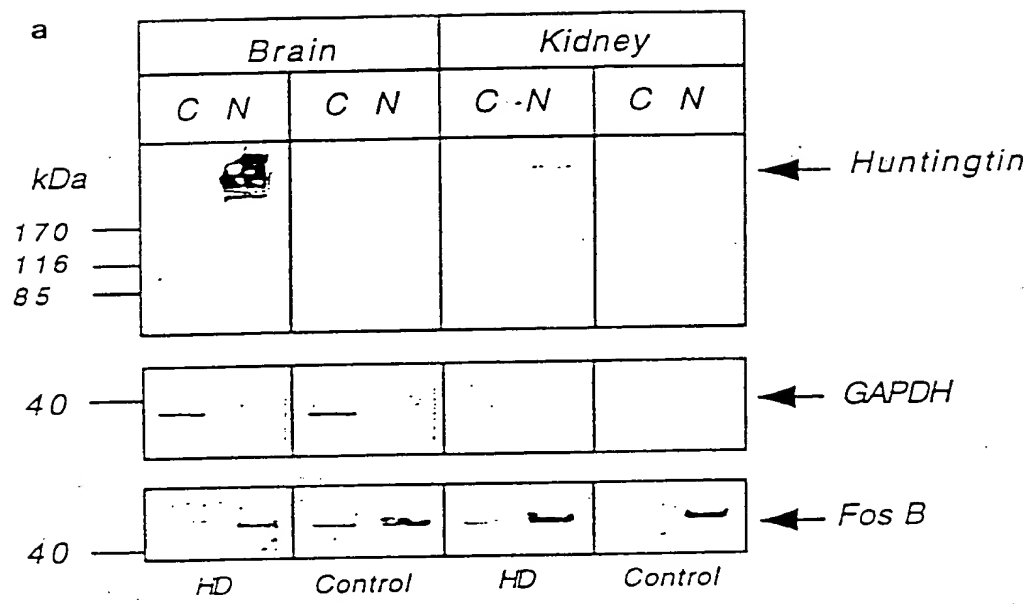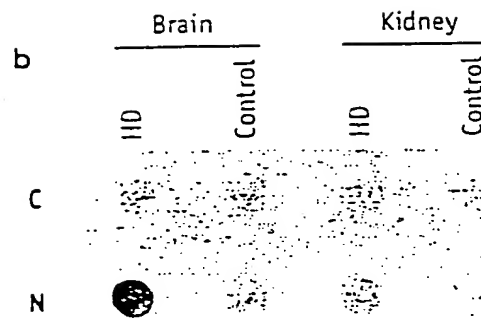
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ABSTRACT

The present invention relates to methods of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on filters. Preferably, said fibrils or aggregates are indicative of a disease, preferably of a neurodegenerative disease such as Alzheimer's disease or Huntington's disease. In addition, the present invention relates to inhibitors identified by the method of the invention, to pharmaceutical compositions comprising said inhibitors and to diagnostic compositions useful for the investigation of said amyloid-like fibrils or aggregates.

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